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The Efflux of a Fluorescent Probe Is Catalyzed by an ATP-Driven Extrusion System in *Lactococcus lactis*

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Many bacteria, both gram positive and gram negative, extrude in an energy-dependent manner the fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5[and -6]-carboxyfluorescein (BCECF) (D. Molenaar, T. Abee, and W. N. Konings, *Biochim. Biophys. Acta* 1115:75–83, 1991). This efflux was studied in detail in *Lactococcus lactis*, and several indications that a transport system is involved were found. This transport system is most likely driven by ATP or a related compound. The evidence is that BCECF extrusion (i) occurs against a BCECF gradient, (ii) is strictly correlated with ATP concentration and not with the proton motive force, and (iii) is inhibited by vanadate and to a lesser extent by *N,N'*-dicyclohexylcarbodiimide. Most convincingly, a UV mutant with a strongly reduced efflux rate was isolated. Such a mutant was isolated from a BCECF-loaded and lactose-energized population by selection of highly fluorescent cells in a flow cytometer-cell sorter. The physiological function of this extrusion system is unknown, but its characteristics classify it among the traffic ATPases.

The fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5[and -6]-carboxyfluorescein (BCECF) is at physiological pH virtually membrane impermeable in many animal cells and artificial membranes. Nevertheless, several bacteria studied, both gram positive and gram negative, exhibit a fast efflux of BCECF. In a previous publication it was proposed that BCECF efflux in *Lactococcus lactis* is mediated by a transport system, since it is dependent on metabolic energy but independent of the proton motive force (24). Efflux proceeds by a first-order process even when intracellular BCECF concentrations are 1 to 3 mM, suggesting that the extrusion system works at these concentrations far below its Michaelis constant (K_m) and is not very specific for BCECF.

In this paper evidence is presented for the existence of an extrusion system and for its energization by ATP or a closely related high-energy compound. Several ATP-driven transport systems are known in bacteria and have been grouped together with similar transport systems from eukaryotes into a class of traffic ATPases, or more generally ATP-binding cassette proteins (18, 23). Among these are a number of transport systems which catalyze efflux of very different compounds. In eukaryotes there are the genetically and biochemically characterized multidrug resistance (Mdr) P-glycoproteins (2, 6, 14) which extrude several chemically unrelated drugs, a chloroquine efflux protein of *Plasmodium falciparum* (10), the yeast α -factor efflux system (3), the suggested heavy metal efflux system from *Leishmania tarentolae* (4), and the biochemically characterized taurocholate transport system of the hepatocyte canalicular membrane (26). In prokaryotes there are the efflux systems for hemolysin, colicin V, and microcin B17 of *Escherichia coli* (7, 9, 11, 16); for leukotoxin from *Pasteurella haemolytica* (37); for cyclolysin from *Bordetella pertussis* (12); for a protease from *Erwinia chrysanthemi* (20); for erythromycin of *Staphylococcus epidermidis* (33); for β -1,2-glucan of *Rhizobium meliloti* and *Agrobacterium tumefaciens* (5, 36); for tylosin from *Streptomyces fradiae* (34); and for daunorubicin and doxorubicin (two mammalian Mdr P-glycoprotein sub-

strates) in *Streptomyces peucetius* (15). The BCECF extrusion system described here is most probably ATP driven and is inhibited by vanadate. It displays at least in these aspects similarity with the eukaryotic Mdr P-glycoprotein.

MATERIALS AND METHODS

Media and strains. *L. lactis* subsp. *lactis* ML3 was grown at 30°C on a modified MRS medium (8), containing (per liter) 10 g of tryptone, 10 g of Lab-Lemco powder (Oxoid, Basingstoke, England), 5 g of yeast extract, 2 g of K_2HPO_4 , 0.2 g of $MgSO_4$, 35 mg of $MnSO_4$, and 0.5 ml of Tween 80, adjusted to pH 6.3 with HCl. The medium was supplemented with separately sterilized 1% lactose or with separately sterilized 1% galactose and 20 mM arginine.

L. lactis subsp. *lactis* IL1403 was grown on the same MRS medium supplied with 1% glucose and 50 mM K-malate, pH 6.5.

Isolation of mutants with reduced BCECF efflux. For UV irradiation mutagenesis, an overnight culture of *L. lactis* subsp. *lactis* ML3 was washed and suspended in 100 mM $MgSO_4$ to a density of 10^7 CFU/ml. The suspension (10 ml) was irradiated with UV light in a petri dish. Irradiation led to a CFU survival of 2% (with an estimated mean chain length of four cells, a cell survival of 0.5% can be expected). The cells were suspended in 200 ml of MRS and grown in the dark until an optical density at 660 nm of approximately 0.6 was reached.

Mutated cells were loaded with BCECF according to the procedure described previously (24). After loading they were resuspended in 50 mM K-phosphate, pH 7, with 1% lactose and left for 30 min at 30°C. Cells were washed again and kept on ice. A flow cytometer was used to separate high-fluorescence and low-fluorescence cells. Approximately 2,300 high-fluorescence events were recorded and separated. This was less than 0.5% of the total events recorded. Of these 2,300 events approximately 600 colonies grew on MRS agar. Thirty-two colonies were tested for BCECF efflux as follows. Colonies were inoculated overnight in 4 ml of MRS medium, of which 100 μ l was transferred to 10 ml of MRS and allowed to grow to an optical density at 660 nm of

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approximately 0.6. These cells were spun down and washed, and half of the cells were loaded with BCECF according to the procedure described elsewhere (24). Subsequently, half of the loaded cells were incubated in Eppendorf tubes for 30 min in 1 ml of 50 mM K-phosphate, pH 7, with 1% (wt/vol) lactose at 30°C. The other half was left on ice and used for reference. After this the cells were spun down, and fluorescence in the pellets was compared on a UV transilluminator used for viewing DNA-ethidium bromide gels. Only two samples showed reduced efflux, indicating that many false-positive cells had been sorted. To obtain more mutants in a less elaborate way, the selected 600 colonies were collected, inoculated, and subjected to a second selection procedure on the flow cytometer. Now 14% of the recorded events showed high fluorescence. Of these newly selected cells, all 32 colonies after culturing and loading with BCECF showed reduced efflux.

Measurement of efflux. Loading of cells was performed as described before (24). The intracellular BCECF concentration is then in the range of 1 to 3 mM. Cells were separated from the medium in an efflux assay, either by filtration or by centrifugation. The centrifugation assay has been described before (24). In this assay, the amount of BCECF in the supernatant is measured and the percentage of BCECF remaining inside is calculated. The filtration assay, in which the amount of BCECF intracellularly is measured directly, gave for unknown reasons more scatter in the time series. It was therefore used only in experiments in which a large extracellular amount of BCECF was present at the start of the experiment. Cells were incubated (0.35 mg of protein per ml), and 200- μ l samples were withdrawn and were spotted on a 0.45- μ m-pore-size cellulose nitrate filter (20-mm diameter; BA85; Schleicher & Schuell) with vacuum applied with a waterjet pump. Immediately the filter was washed twice with 2 ml of ice-cold 50 mM K-phosphate, pH 7. Filters were put in test tubes, 3 ml of 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane-Cl (bis-Tris-propane-Cl) (pH 9.1) with 0.1% Triton X-100 was added, and cells were resuspended by shaking vigorously. This suspension was incubated overnight at 4°C in the dark to let BCECF leak out. Debris was removed by centrifugation, and fluorescence was measured with wavelengths for excitation at 502 nm and emission at 525 nm and with slit widths of 10 and 15 nm, respectively. Fluorescence was assumed to be proportional to the BCECF concentration. It was confirmed that BCECF did not bind to the filters. The internal volume was assumed to be 2.9 μ l/mg of cell protein (29).

Measurement of intracellular ATP concentration. The cell suspension was mixed with half a volume of 14% perchloric acid–9 mM Na₂-EDTA. The supernatant was neutralized with half a volume of 1 M KOH–1 M KHCO₃. This neutralized extract was diluted 40-fold in 40 mM Tris-SO₄–2 mM EDTA, pH 7.75. ATP was measured with the firefly luciferase assay as described by Otto et al. (28).

Materials and instrumentation. BCECF was obtained from Molecular Probes, Inc., Eugene, Oreg. A stock suspension of 5 mg/ml was made in water, which was first brought to approximately pH 11 with KOH, and subsequently after a few minutes was neutralized with HCl, as was recommended by the supplier. The solution was stored at –20°C and kept in the dark.

The fluorometer was a Perkin-Elmer LS 50 with computer-controlled data acquisition and storage. The flow cytometer-cell sorter was a FACSTAR 400.

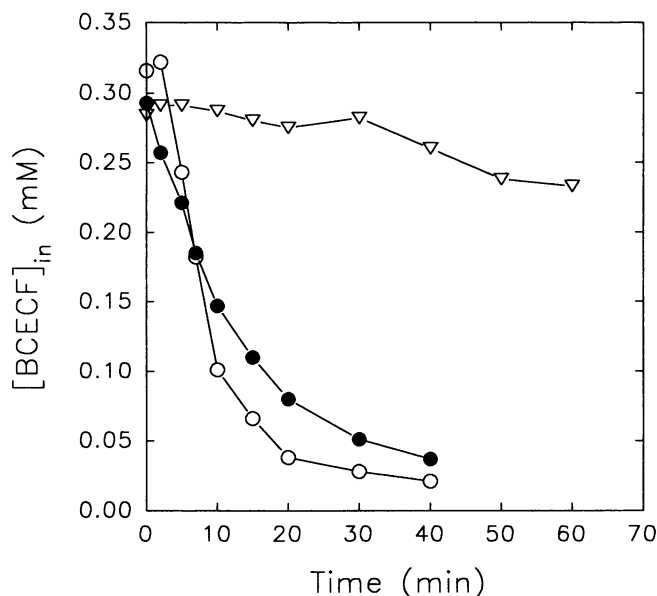


FIG. 1. Efflux of BCECF against a concentration gradient. Cells (*L. lactis* ML3) were loaded with BCECF and resuspended in 50 mM K-phosphate, pH 7, with 1 mM BCECF. Efflux was monitored in the absence of an energy source (∇), in the presence of 3 mM lactose (●), or in the presence of lactose and the ionophores valinomycin (0.5 μ M) and nigericin (1 μ M) (○). The temperature was 30°C.

RESULTS

Efflux against a high concentration gradient. To determine whether BCECF efflux is driven by metabolic energy, efflux was studied in the presence of a high extracellular BCECF concentration (Fig. 1). The extracellular concentration in this experiment was 1 mM. In the absence of an energy source efflux occurred at a very low rate, while in the presence of lactose as an energy source efflux was observed to proceed at a high rate against a concentration gradient. Steady state was not reached in this experiment, but the concentration gradient was at least 40-fold. The rate of efflux was hardly affected or not affected by the addition of the combination of valinomycin and nigericin, indicating that efflux is not dependent on the proton motive force. The small stimulation observed in Fig. 1 upon addition of these ionophores is due mainly to experimental variations in different batches of cells used in these experiments. The results suggest that BCECF efflux is mediated by an active transport system.

Temperature dependence of BCECF efflux. *L. lactis* subsp. *lactis* ML3 was loaded with BCECF, and efflux at an extracellular pH of 6 was monitored by the method described earlier (24). In this method the decrease of the fluorescence signal at a constant intracellular pH is used to calculate the first-order efflux constant. The efflux constant was measured at temperatures ranging from 14.4 to 32.0°C. The efflux constants which showed a marked temperature dependence are plotted in an Arrhenius plot (Fig. 2). Two linear regions are observed, with a transition temperature of 25.5°C. At high temperatures an activation energy of 35.5 kJ/mol is measured, and at low temperatures this value is 110 kJ/mol.

Inhibition of BCECF efflux. Support for the claim that BCECF efflux is mediated by a transport system can be

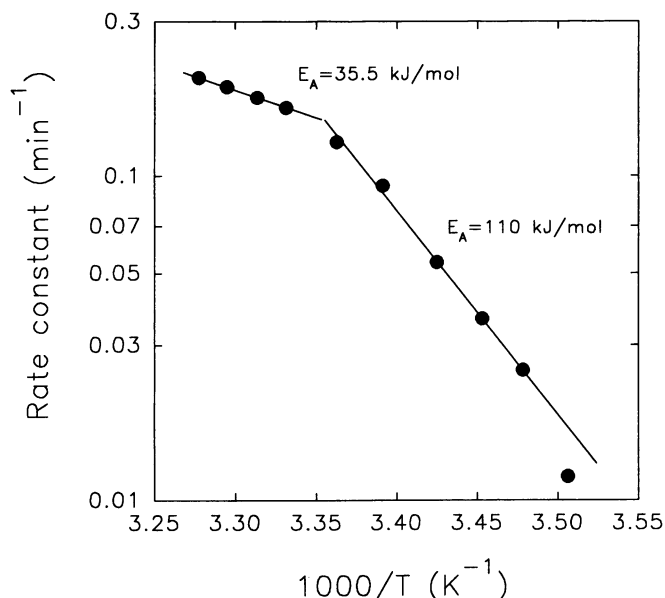


FIG. 2. Arrhenius plot of BCECF efflux. BCECF-loaded cells (*L. lactis* ML3) were resuspended in 50 mM K-phosphate buffer, pH 6, with 3 mM lactose, and efflux was measured on the fluorometer. Two phases are visible in the plot. The activation energies calculated from the fitted lines (E_A) are indicated for both phases. The vertical axis has a logarithmic scale.

obtained by demonstrating that BCECF efflux is inhibited by certain compounds. First some experiments were done with nonenergized cells. As is shown above (Fig. 1), the rate of efflux is low under these circumstances. However, this efflux takes place against the BCECF concentration gradient and thus must be mediated by a transport system. Figure 3 shows

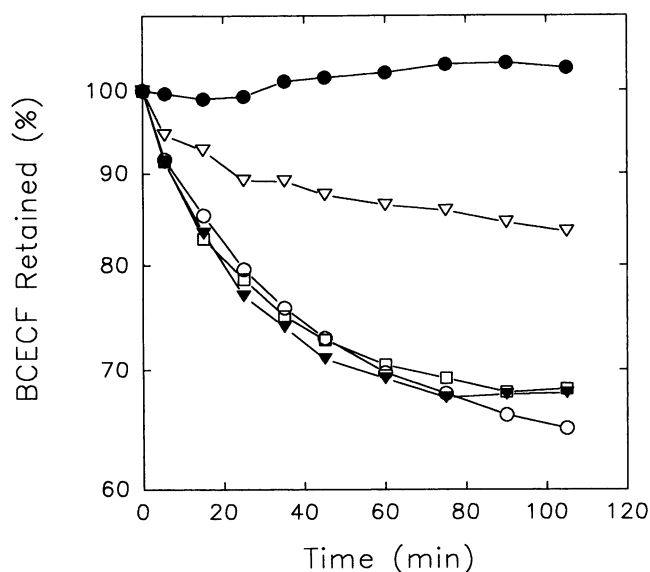


FIG. 3. Inhibition of BCECF efflux in nonenergized cells. Cells (*L. lactis* ML3) were resuspended in 50 mM K-phosphate, pH 7. Additions were none (○), 0.5 mM sodium-vanadate (●), 250 μM DCCD (▽), 20 μM mersalyl (▼), or 0.5 mM sodium-arsenate (□). The temperature was 30°C. The vertical axis has a logarithmic scale.

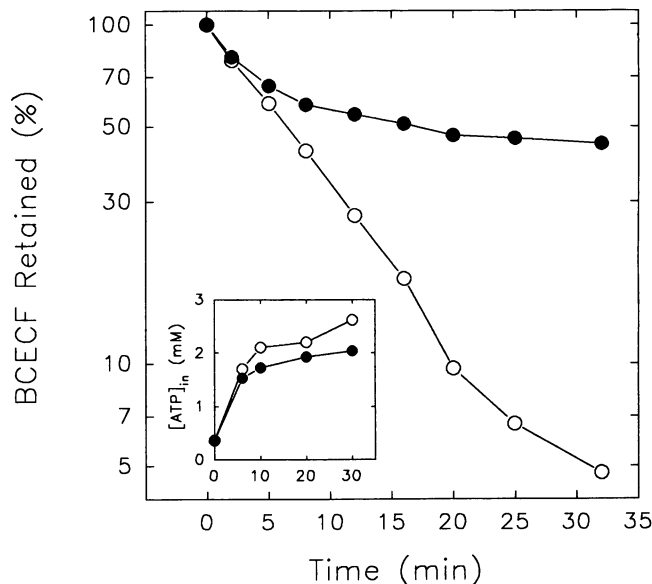


FIG. 4. Inhibition of BCECF efflux by vanadate in arginine-energized cells. BCECF-loaded cells were washed and resuspended in 50 mM K-HEPES–50 mM KCl, pH 7, without (○) or with 0.5 mM sodium-vanadate added (●). They were preincubated on ice in this buffer for 30 min and then transferred to 30°C, at which point 5 mM arginine was added. The vertical axis has a logarithmic scale. The inset shows the time course of the intracellular ATP concentration during these experiments.

the effect of different compounds on the efflux under nonenergized circumstances. Vanadate inhibits efflux completely at 0.5 mM, whereas 250 μM *N,N'*-dicyclohexylcarbodiimide (DCCD) has a less pronounced effect. The effects of vanadate, arsenate, and DCCD were also tested in a K–4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer (25). In the phosphate-free buffer, arsenate inhibited efflux only slightly while vanadate inhibited efflux by about 90%. Inhibition by DCCD was comparable to the results shown in Fig. 3. The ATP content of these cells varied between 0.3 and 0.5 mM, but a correlation with the rate of efflux was not observed under these circumstances, indicating that inhibition was probably not due to energy depletion.

The effect of vanadate was also investigated under energized conditions. It is important to ascertain that vanadate does not interfere with energy generation. Glycolysis in *L. lactis* is inhibited by vanadate, but the arginine deiminase route is not, even after 30 min of incubation in the presence of 0.5 mM vanadate. This is concluded from the lack of inhibition of the rate of ammonium production measured with a pH electrode and the maintenance of the electrical membrane potential as measured with a tetraphenylphosphonium ion electrode (results not shown) (21, 35). Also, the ATP pool was not significantly reduced by vanadate during arginine energization (Fig. 4, inset). Therefore, the effect of vanadate on BCECF efflux could be monitored in arginine-energized cells. This was done in a phosphate-free buffer (K-HEPES). Vanadate (0.5 mM) started to inhibit after 5 min, after which the inhibition gradually increased to approximately 95%, as can be deduced from the tangents to the curves, of which the slopes are proportional to the efflux rate constant (Fig. 4). In a phosphate buffer, inhibition by 0.5 mM vanadate started after 15 min and increased up to 90% after 30 min (25).

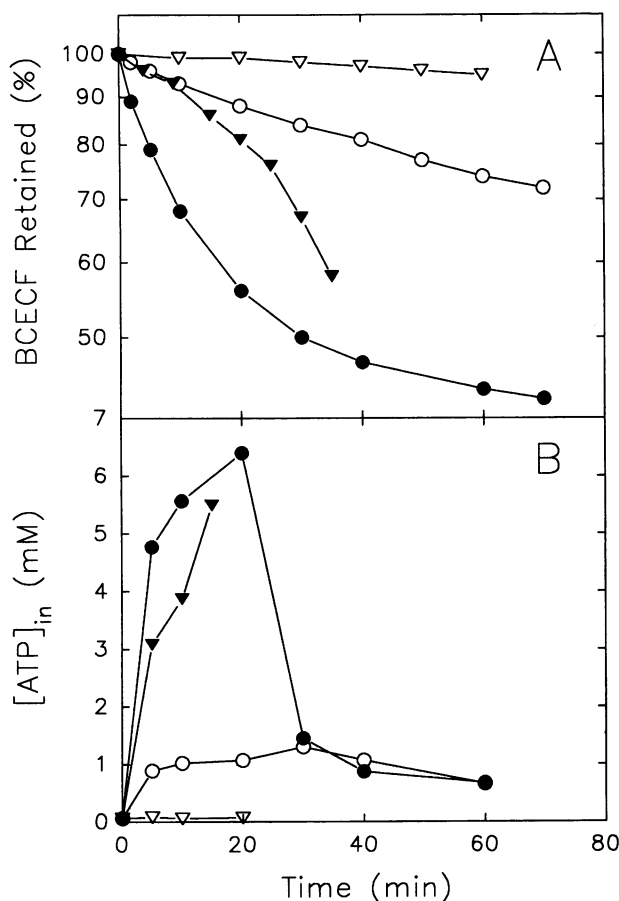


FIG. 5. Correlation of intracellular ATP concentration and rate of BCECF efflux. *L. lactis* IL1403 was loaded with BCECF, resuspended in 50 mM K-phosphate, and left to efflux under the following conditions: no additions, pH 7 (▽); 25 mM glucose, pH 7 (▼); 30 mM K-malate, pH 7 (○); or 30 mM K-malate, pH 5 (●). Prior to energization malate-energized cells were loaded with 50 mM K-lactate for 30 min at pH 7. (A) BCECF efflux (the vertical axis has a logarithmic scale); (B) intracellular ATP concentration. The temperature was 30°C.

Correlation of BCECF efflux with intracellular ATP. Since the independence of efflux from the proton motive force and the inhibition by vanadate indicated an involvement of a phosphorylated high-energy metabolite, the effect of different cytoplasmic ATP concentrations was investigated. This was done in *L. lactis* subsp. *lactis* IL1403, which can be energized by malolactic fermentation (32). Metabolic energy generation by malolactic fermentation is not achieved at the level of substrate phosphorylation but is achieved at the level of proton motive force generation by electrogenic lactate/malate antiport and by the consumption of protons in the cytoplasm during malate decarboxylation (31). ATP is formed indirectly, through the reverse action of the proton-translocating ATPase, driving ATP synthesis at the expense of the proton motive force. Since the proton motive force of malate-metabolizing cells is higher at low medium pH, malate energization yields higher intracellular ATP concentrations at low medium pH compared with those at high medium pH. Figure 5 shows that there is a positive correlation between the intracellular ATP concentration and the rate of BCECF efflux, irrespective of the mode of energy

generation. The electrical membrane potential was at pH 7 the same in cells energized with glucose or malate. Nevertheless, the efflux rates differed drastically. Efflux rates were highest in cells energized with malate at pH 5 and correlated with the intracellular ATP concentrations under those conditions. The drop in ATP content after 20 min of malate energization at pH 5 is probably due to the inhibition of malate/lactate exchange caused by the increased external lactic acid concentration. It should be mentioned that the efflux in strain IL1403 appears to be lower in general compared with efflux in strain ML3.

Reduced efflux in a mutant. UV mutants of *L. lactis* subsp. *lactis* ML3 with reduced BCECF efflux were isolated by selection in a flow cytometer with a cell sorter. In the first selection of approximately 600 cells, two mutants with reduced BCECF efflux were found in 32 colonies tested. The mutants showed different phenotypes. One mutant had not only a reduced BCECF efflux but also a strongly reduced growth rate and a 70% reduced rate of glycolysis, as deduced from medium acidification of washed suspensions observed with a pH electrode. The second mutant had a growth rate comparable to that of the wild type and a normal rate of glycolysis. Its BCECF efflux rate was reduced but was not zero. The repeated selection on a culture grown from the first selection of cells yielded only BCECF efflux mutants of the second type. Twelve mutants were tested in detail with respect to the rate of BCECF efflux. The efflux rate constants were determined as described before (24). All mutants showed approximately the same reduction in efflux rate, and some residual efflux activity was observed in all cases. One of these efflux-negative mutants, termed *Bef*⁻ (BCECF efflux negative), was further characterized. Its rate of glycolysis, ATP content, and electrical membrane potential as observed with a tetraphenylphosphonium ion electrode were comparable to those of the wild type. Efflux in this mutant was still energy dependent. However, the energy-dependent efflux of BCECF was reduced by 80% compared with that of the wild type (Fig. 6), and the nonenergized *Bef*⁻ showed no detectable efflux over a period of 2 h.

Resistance of the wild type and mutants to several compounds. Since some similarity was observed between the BCECF efflux pump and the eukaryotic Mdr P-glycoprotein, the resistance of the efflux mutant and the wild-type strains against several compounds were compared. To this end, a filter disc assay was used. Discs of filter paper (Whatman no. 3) were loaded with 20 μ l of a highly concentrated solution of the compound, dried, and placed on a top agar in a petri dish with a confluent plated culture. The following compounds were tested: the antibiotics kanamycin, chloramphenicol, tetracycline, and erythromycin; the BCECF analogs fluorescein and carboxyfluorescein; the Mdr P-glycoprotein substrates and inhibitors daunomycin, vinblastin, verapamil, and actinomycin D; the substrates of *P. falciparum* multidrug resistance quinine and chloroquine; the uncouplers carbonylcyanide *m*-chlorophenylhydrazide and dinitrophenol; the heavy metals Cu²⁺ and Pb²⁺; the bacterial multidrug resistance substrates (27) rhodamine 6-G and ethidium; and DCCD, tetraphenylphosphonium ion, vanadate, and arsenate. No significant difference in the sizes of the halos could be observed between the wild type and mutant with any of these compounds. With the filter disc assay as presented here, moderate differences in resistance may not be detected. Therefore, the effects of different concentrations, in the range in which they inhibited growth, of daunomycin, quinine, chloroquine, ethidium, and DCCD were studied.

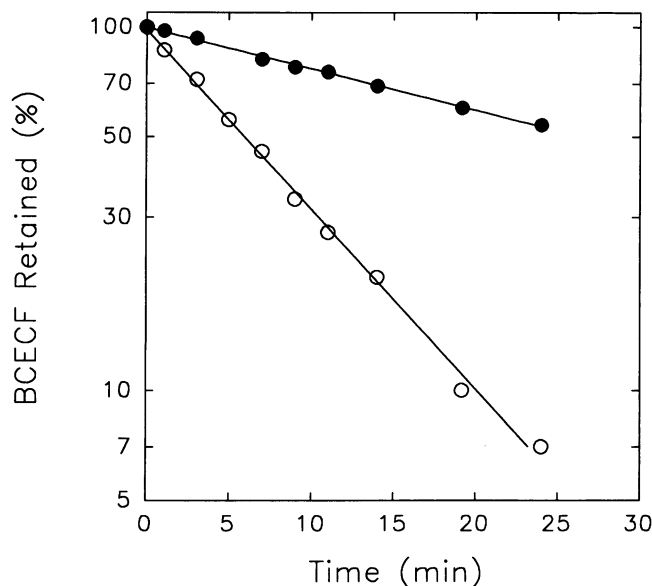


FIG. 6. Comparison of BCECF efflux in the wild type and a mutant. Cells of the ML3 wild type (○) and of *Bcf⁻* (●) were loaded with BCECF and resuspended in 50 mM K-phosphate, pH 7, with 10 mM lactose. The half times calculated from the curves are 6 min for the wild type and 26 min for *Bcf⁻*. The vertical axis has a logarithmic scale. The temperature was 30°C.

However, no differences in growth rates between the wild type and mutant were detected.

DISCUSSION

Several mechanisms can be considered for the fast BCECF efflux observed in *L. lactis*.

(i) **Passive diffusion.** A passive diffusion process, in which the membrane is permeable for BCECF, is for several reasons not very likely to be the case. First, BCECF is a large molecule with a hydrophilic character at physiological pH and negative charges distributed over the molecule. Also, carboxyfluorescein, the compound from which BCECF is derived and which is thought to be more hydrophobic than BCECF, leaks only very slowly from artificial membrane vesicles (38). In addition, BCECF efflux from most animal cells is relatively slow (32a).

Although it is not conclusive evidence, the activation energies found for efflux in Fig. 2 are more in agreement with a carrier-mediated process, which is in the range 7 to 20 kcal/mol (ca. 30 to 84 kJ/mol) (19), than with passive diffusion. The transition in the Arrhenius plot could reflect a transition in the membrane, e.g., the formation of lipid patches (19). However, it may also reflect the temperature dependence of energy generation, which may limit the efflux rate below 25.5°C. The inhibition of efflux with vanadate and DCCD is also in agreement with the hypothesis that efflux is not simple diffusion through the membrane but is a carrier-mediated process. The complete inhibition with vanadate (Fig. 3) even supports the hypothesis that the cytoplasmic membrane is itself impermeable to BCECF under physiological conditions.

(ii) **Facilitated diffusion.** The BCECF efflux against up to at least a 40-fold concentration gradient shows that some form of metabolic energy must be involved. Efflux of BCECF could be driven by the difference in composition of cyto-

plasm and medium, since the apparent pK_a of intracellular BCECF is 0.3 higher than that of BCECF in 50 mM K-phosphate buffer (24). If the protonated species of the carboxylic group corresponding to this pK_a were the diffusing species, either facilitated or nonfacilitated, the pK_a difference could drive efflux against a concentration gradient. However, the concentration ratio would then at most be 2 in the presence of valinomycin and nigericin, assuming that intracellular and extracellular pHs are equal.

(iii) **Secondary active efflux.** A secondary active efflux system driven by the electrical membrane potential or the chemical gradient of another compound can be ruled out, since the abolishment of the electrical and pH gradients has a negligible effect on the rate of efflux. This also shows that BCECF efflux is not mediated by a proton motive force-dependent transport system extruding cationic dyes and related compounds (22) or by a bacterial multidrug resistance-like carrier, which is probably an H^+ /drug antiporter (27). In *L. lactis* a similar system extruding ethidium, which unlike BCECF efflux was inhibited by a collapse of the pH gradient with nigericin, was found (25).

(iv) **ATP-driven efflux.** The positive correlation of the efflux rate with intracellular ATP concentration and the inhibition by vanadate point to the involvement of a high-energy phosphorylated compound, most likely ATP itself, in the catalytic cycle of this carrier (13). The same conclusion was drawn for the Mdr P-glycoprotein, which is also specifically inhibited by vanadate (17). The relation between the intracellular ATP concentration and efflux rate is not unique, as can be seen in Fig. 5. For example, in the first 10 min glucose- and malate-energized cells at pH 7 have the same rate of BCECF efflux, although the ATP concentration is much higher in glucose-energized cells. The explanation for this could be that the concentrations of products in the catalytic cycle of the efflux system, e.g., ADP and phosphate, also play a role in determining the efflux rate. These concentrations can be different and will change in time with the different modes of energization.

Estimations can be made for the kinetic constants of the efflux system for BCECF efflux under physiological conditions (lactose energization and pH 7). A lower limit of the V_{max} of this carrier can be indicated, when it is realized that the first-order rate constant for efflux (k) is equal to V_{max}/K_m , assuming that the carrier obeys Michaelis-Menten kinetics. Taking a lower limit for K_m of 3 mM (24), a specific intracellular volume of 2.9 μ l/mg of cell protein (29) (K_m can then equivalently be expressed as 8.7 nmol/mg of cell protein), and a half-life of 6 min ($k = 0.116 \text{ min}^{-1}$), a lower limit for V_{max} will then be 1 nmol/min/mg of cell protein. This is 1 order of magnitude lower than V_{max} s for (for example) amino acid carriers of *L. lactis* (30).

What is the physiological function of this carrier? Recalling the functions known for the presently characterized ATP-driven efflux systems mentioned in the introduction, one might think of a multidrug resistance-like system or a system involved in the excretion of peptides, small proteins, or specific organic compounds, like polysaccharides. Of the known extrusion systems, only the Mdr P-glycoprotein has a demonstrated broad substrate range. To our knowledge, BCECF does not resemble any naturally occurring compound, and it is hard to imagine that the extrusion system is specific for BCECF only. Therefore, the extrusion system probably has affinity for chemically unrelated compounds. It could be related to the Mdr P-glycoprotein and function as an extrusion system for potentially toxic xenobiotics. However, since mutants show no aberrant behavior in physiol-

ogy, growth rate, and resistance to several compounds, a xenobiotic extrusion function seems unlikely but cannot be excluded. A direct kinship with Mdr P-glycoprotein is unlikely, since mutants were not inhibited more than the wild type by Mdr P-glycoprotein substrates. In eukaryotic cells metabolic energy-dependent BCECF efflux has also been described (1), and from inhibitor studies it was concluded that efflux was probably not catalyzed by the Mdr P-glycoprotein.

It should be mentioned that no mutants with totally absent BCECF efflux were found and that efflux in the mutants found was still metabolic energy dependent. This could point to the presence of a second efflux system but more likely to a mutation affecting, for example, only the affinity of the system for BCECF. The failure to isolate 100% reduced efflux mutants may indicate the lethality of such a mutation and consequently the essentiality of the system for life. On the other hand, the selection procedure, with growth steps in between, may not have allowed the isolation of such mutants. It is also peculiar that in many bacteria tested, both gram positive and gram negative, BCECF extrusion was observed (24). Our present attempts to clone the gene(s) for this extrusion system will, it is hoped, shed more light on its physiological function and possible relation to the traffic ATPases.

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